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Anxiolytic-like effect of etazolate, a type 4 phosphodiesterase inhibitor in experimental models of anxiety

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Etazolate is a selective inhibitor of type 4 phosphodiesterase (PDE4) class enzyme. Antidepressant-like effect of etazolate has been previously demonstrated in the rodent models of depression. The present study was designed to investigate the anxiolytic-like activity of etazolate in experimental mouse models of anxiety. The putative anxiolytic effect of etazolate (0.25-1 mg/kg, ip) was studied in mice by using a battery of behavioural tests of anxiety such as elevated plus maze (EPM), light/dark (L/D) aversion, hole board (HB) and open field (OFT) with diazepam (2 mg/kg, ip) as reference anxiolytic. Like diazepam (2 mg/kg, ip), etazolate (0.5 and 1 mg/kg, ip) significantly increased the percentage of both time spent and entries into open arms in the EPM test. In the L/D test etazolate (0.5 and 1 mg/kg, ip) increased the both total time spent in and latency time to leave the light compartment. Etazolate (0.5 and 1 mg/kg, ip) also significantly increased head dipping scores and time spent in head dipping, whereas significantly decreased the head dipping latency in HB test. In addition, etazolate (0.5 and 1 mg/kg, ip) significantly increased the ambulation scores (square crossed) and number of rearing in OFT. In conclusion, these findings indicated that etazolate exhibited an anxiolytic-like effect in experimental models of anxiety and may be considered an alternative approach for the management of anxiety disorder.

Keywords: Anti-anxiety, Etazolate, Phosphodiesterase 4

Anxiety, an emotional state is one of the most frequently occurring psychiatric disorder across the globe[1]. Moreover, anxiety disorders associate with significant disability which has a negative impact on the quality of life. Despite a steady increase in the development of anxiolytic drugs, the prevalence of the disorder remains stable that could be attributed to the unclear neurobiological understanding of pathophysiology or the inconsistent efficacy of current pharmacological treatment. Benzodiazepine (BZD) class compounds are generally prescribed medications for the treatment of several forms of anxiety but this class compounds have prominent side effects, such as sedation, dependence, cognitive and psychomotor impairment[2,3]. Thus, the awareness that BZDs have a narrow safety margin, has promoted many researchers to evaluate new agents with a balance of high efficacy and less adverse effects that could be useful for anxiety treatment.

Several reports provided evidences to support that a dysfunction of the cyclic AMP (cAMP) signaling may be implicated as promising mechanism in the pathophysiology of anxiety disorders[4]. Moreover, studies have been shown that cAMP triggers protein kinase A (PKA)-mediated phosphorylation of the cAMP response element binding protein (CREB), which in turn activates intracellular signaling cascade that implicates in the pathophysiology of anxiety disorder[4]. This hypothesis provides a frame work in which the pathophysiology and pharmacotherapy for anxiety may congregate on the modulation of cAMP signaling. In this respect, agents are expected to influence cAMP intracellular cascade such as PDE4 inhibitors may act as a potential strategy to treat anxiety disorder.

Phosphodiesterases (PDEs) are a diverse group of metallophosphohydrolases enzymes and comprise eleven families of enzymes (PDE1–PDE11), responsible for the hydrolysis of the secondary messenger viz. cAMP and cGMP[5,6]. Of the eleven PDE families, PDE4 family enzymes are highly specific for cAMP and proven to be of particular importance in neuro-psychopharmacology[7,8]. PDE4, an enzyme catalyzes the cAMP hydrolysis and regulates its intracellular concentration[6,8]. Previous studies have been addressed the anxiolytic-like effect of rolipram, a PDE4 inhibitor in experimental models of anxiety such as EPM, L/D aversion and HB tests by enhancing cAMP signaling[9,10].

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Etazolate is a pyrazolopyridine class compound which belongs to a family of molecules with PDE4 enzyme inhibitor activity[11,12]. Preclinical studies as well as pharmacokinetic and safety profiles in Phase I and Phase IIa of clinical studies have established that etazolate is a well-tolerated drug devoid of major side effects[12]. Several studies investigated that etazolate could be a lead candidate for the treatment of Alzheimer's disease[12]. Etazolate produced an antidepressant-like effect in the animal models of depression[13]. Considering the findings mentioned previously that cAMP signaling is implicated in the pathophysiology of anxiety disorders and the fact that etazolate plays an important role in the modulation of cAMP signaling, the present study has been designed to investigate the anxiolytic potential of etazolate.

Material and Methods

Animals—Behavioural based experiments were carried out using Swiss Albino mice (20–25 g), procured from Hisar Agricultural University, Haryana, India. Animals were kept in polypropylene boxes under standard laboratory conditions (23 ± 2 °C and 60 ± 10% RH), and maintained on 12:12 h light/dark cycle. Standard diet and filtered water were given *ad libitum*. All the experiments were carried out between 09.00–14.00 hrs. in accordance with the Institutional Animal Ethics Committee of Birla Institute of Technology & Science, Pilani, India (Protocol No. IAEC/RES/14/03) guidelines.

Drugs and treatments—Etazolate hydrochloride was procured form Tocris Bioscience, UK. Diazepam was purchased from Cipla Ltd. India. Etazolate and diazepam were dissolved in distill water and always freshly prepared before administration. Etazolate (0.25-1 mg/kg, ip) and diazepam (2 mg/kg, ip) were administered to mice 30 min prior to behavioural observations. The dose range of etazolate was selected according to Jindal *et al.*[13] previous study performed in our laboratory.

Elevated plus maze—The EPM test was first evaluated for rats[14] and later adapted for mice[15]. In brief, the apparatus consisted of a wooden maze with two enclosed arm (30 × 5 × 15 cm^3) and two open arms (30 × 5 × 0.25 cm^3) that extended from a central platform (5 × 5 cm^2) to form a plus sign. The plus-maze apparatus was elevated to a height of 45 cm and placed inside a sound-attenuated room. The trial was started by placing a mouse on the central platform of the maze facing its head towards an open arm. The behavioural performances recorded during a 5 min test period were: percentage open arms entries, percentage time spent in open arms and total entries. Entry into an arm was considered valid only when all four paws of the mouse were inside that arm[16]. The animal activities were tracked and recorded by an overhead video camera and *smart version 2.5* computer software (Panlab co., USA). The apparatus was thoroughly cleaned with 70% ethanol after each trial.

Light/dark aversion test—The L/D apparatus comprised of a box divided into two separate compartments, occupying two-thirds and one-third of the total size, respectively. The larger compartment (light compartment) was illuminated by a 60-watt bulb, while the smaller (dark compartment) was entirely black and enclosed under a dark cover. The L/D compartments were separated by a partition with a tunnel to allow passage from one compartment to the other[17]. At the beginning of the test, the mouse was placed individually at the center of the light compartment facing towards the tunnel and was allowed to explore the entire apparatus for 5 min. The behavioural parameters such as latency time to leave the light compartment, total time spent in the light compartment and number of transitions between the L/D compartments were tracked and recorded using computer software *smart version 2.5* (Panlab Co., USA). A compartment entry was considered valid when all the four paws of mouse were inside that chamber. The apparatus was thoroughly cleaned with 70% ethanol after each trial.

Hole board test—The HB apparatus consisted of a grey Plexiglas platform (40 × 40 cm^2) raised to a height of 15 cm from the floor of a gray wooden box (40 × 40 × 40 cm^3). The grey Plexiglas platform consisted of 16 equivalent square compartments (12 peripheral and 4 central), each featuring a central circular hole (3 cm diameter). Test session was started by placing each animal in the center of the HB and allowed to freely explore on the apparatus for 5 min. The behavioural performances such as number of head dipping, total time spent in head dipping and latency to the first head dipping[18] were tracked and recorded using computer software *smart version 2.5* (Panlab Co., USA).

Open field test—The apparatus consisted of a wooden box (60 × 60 × 30 cm^3) with the floor divided into 16 squares (15 × 15 cm^2) by black parallel and intersecting lines. The apparatus was illuminated by a 60-watt bulb suspended 100 cm

above. At the beginning of the test, the mouse was placed individually at the center of the square arena. The ambulation scores (number of square crossed) and rearing number (standing upright on the hind legs) were recorded using computer software *smart version 2.5* (Panlab co., USA) for 5 min period. After each individual test session the floor was thoroughly cleaned with 70% ethanol.

Statistical analysis—All values were expressed as mean ± SE. The data obtained from various groups were statistically analyzed using one way analysis of variance (ANOVA) followed by the *post-hoc* Dunnett's test in Graph pad prism 3. The *P* value < 0.05 was considered to be statistically significant.

Results

Elevated plus maze—The effects of etazolate (0.25-1 mg/kg., ip) and diazepam (2 mg/kg, ip) on the behaviour of mice in the EPM are presented in Table 1. Acute treatment with etazolate (0.5 and 1 mg/kg, ip) and diazepam (2 mg/kg, ip) significantly increased the percentage of both open arms entries [F (4, 35) = 9.27, $P < 0.05$] and time spent in open arms [F (4, 35) = 10.89, $P < 0.05$] as compared to control group (Table 1). In addition, etazolate (0.5 and 1 mg/kg, ip) and diazepam (2 mg/kg, ip) significantly decreased the time spent [F (4, 35) = 7.22, $P < 0.05$] in closed arms as compared to control group (Table 1). However, lower dose of etazolate (0.25 mg/kg, ip) had no significant effect on the behavior of mice in EPM (Table 1).

Light/dark aversion test—Etazolate (0.5 and 1 mg/kg, ip) and diazepam (2 mg/kg, ip) treatment significantly increased the latency time to leave the light compartment [F (4, 35) = 10.74, $P < 0.05$] and time spent in light compartment [F (4, 35) = 19.25, $P < 0.05$] as compared to control group (Fig. 1a and 1b). In addition, diazepam significantly increased the number of crossings [F (4, 35) = 3.25, $P < 0.05$] between the compartments, whereas no significant effect on crossings was observed in the etazolate treatment groups (Fig. 1c). Lower dose of etazolate (0.25 mg/kg, ip) did not produce significant change in any of the parameters.

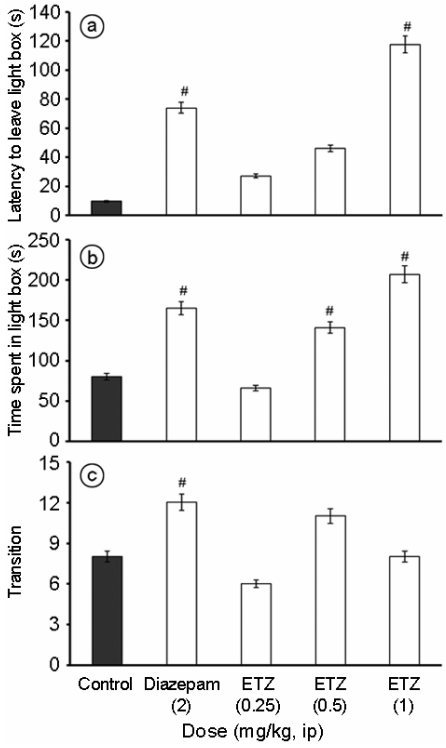

Fig. 1—Effect of etazolate (0.25-1 mg/kg, ip) and diazepam (2 mg/kg, ip) on the behaviour of mice in the light/dark test; (a) latency to leave the light box (s), (b) time spent in the light box (s) and (c) transitions number between the two compartments. Results are expressed as mean ± SE, #$P < 0.05$ when compared with control group. ETZ = etazolate.

Table 1—Effect of etazolate on the behavior of mice in elevated plus maze test
[Values are mean ± SE from 8 animals in each group]

Groups	Dose (mg/kg, ip)	No. of entries		Time spent (s)		OAE (%)	TSOA (%)
		open arm	closed arm	open arm	closed arm		
Control	0	2.12±0.61	5.62±0.50	64.25±9.33	236.75±5.34	27.62±6.89	26.41±2.71
Diazepam	2	6.80±1.46*	4.40±0.87	132.4±10.19*	167.60±10.78*	60.15±2.56*	44.13±3.6*
ETZ	0.25	2.33±0.67	4.50±1.01	64.83±12.14	235.17±10.11	25.17±7.51	21.61±4.04
	0.5	5.29±1.08*	5.86±1.03	111.14±5.52*	188.86±8.95*	48.26±5.53*	37.05±2.98*
	1	5.14±1.52*	4.71±1.26	136.85±11.29*	164.85±7.92*	52.32±3.66*	45.67±6.09*

#$P < 0.05$ when compared with control group (one way ANOVA followed by Dunnett's test);
OAE (%) = percentage of open arms entries; TSOA(%) = percentage of time spent in open arms in seconds; ETZ = etazolate

Hole board test—The results of the HB test are shown in Fig. 2 a-c. Etazolate (0.5 and 1 mg/kg, ip) and diazepam (2 mg/kg, ip) treatment significantly increased the number of head dipping [F (4, 35) = 7.23, $P < 0.05$] and time spent in head dipping [F (4, 35) = 38.79, $P < 0.05$] as compared to control group (Fig. 2a and 2b). In addition, etazolate and diazepam treatment significantly [F (4, 35) = 2.77, $P < 0.05$] decreased the head dipping latency (Fig. 2c). However, etazolate at a dose of 0.25 mg/kg did not produce significant change in any of the parameter as compared to control group.

Open field test —Etazolate (0.5 and 1 mg/kg, ip) and diazepam (2 mg/kg, ip) treatment significantly increased the number of square crossed [F (4, 35) = 7.23, $P < 0.05$] and rearing numbers [F (4, 35) = 38.79, $P < 0.05$] as compared to control group (Fig. 3a and 3b). However, lower dose of etazolate had not significant effect in OFT as compared to control group.

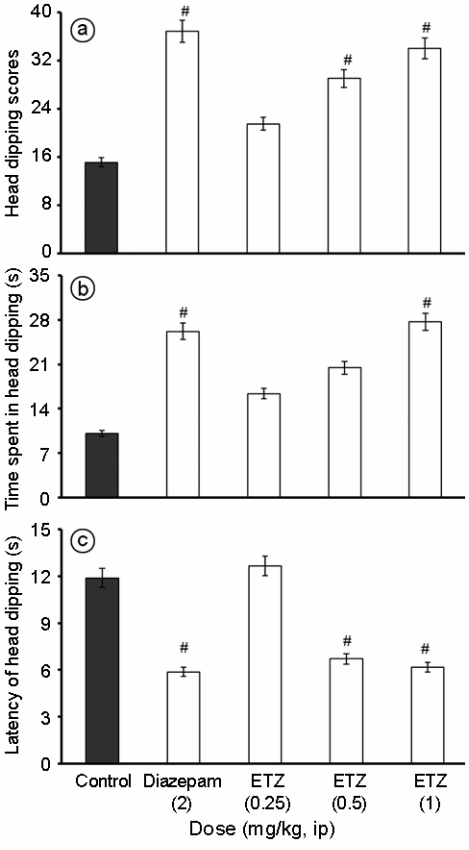

Fig. 2—Effects of etazolate (0.25-1 mg/kg, ip) and diazepam (2 mg/kg, ip) on the behaviour of mice in the hole-board test; (a) head dipping scores, (b) time spent in head dipping (s) and (c) latency of head dipping (s). Results are expressed as mean ± SE, #$P < 0.05$ when compared with control group. ETZ = etazolate.

Out of the three doses (0.25, 0.5 and 1 mg/kg) of etazolate studied in the present investigation, the highest dose (1 mg/kg) was found to be comparatively most effective with respect to all the behavioural parameters, except the effect on transition number as mentioned above. Moreover, at this dose the effect of etazolate was quite similar to diazepam, used as standard anxiolytic in all four tests.

Discussion

In the present study, anxiolytic effect of etazolate was examined in experimental models of anxiety such as the elevated plus maze, light/dark, hole board and open field tests[18]. The results of the present study verify the designed hypothesis that inhibitors of PDE4 enzyme may produce the anxiolytic-like effect. Although, it is uncertain that any single animal model captures all of the components of complex expression of anxiety, thus a battery of tests has been used to evaluate the potential anxiolytic-like effect of etazolate.

EPM is considered as one of the well established model for unconditioned anxiety to detect anxiolytic/anxiogenic-like activity by investigating aspects of physiological and pharmacological behaviour. In the EPM test, increase in both number of entries and time spent into the open arms are the most reliable indicators of decrease anxiety or indicating the anxiolytic-like activity of a compound, while anxiogenic substances have the opposite effect[13,19].

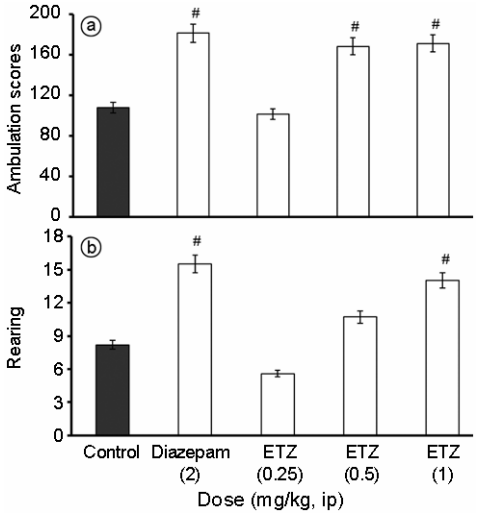

Fig. 3—Effects of etazolate (0.25-1 mg/kg, ip) and diazepam (2 mg/kg, ip) on the behaviour of mice in the open field test; (3a) Ambulation scores, (3b) rearing number. Results are expressed as mean ± SE, #$P < 0.05$ when compared with control group. ETZ = etazolate.

In the present study, treatment with etazolate produced anxiolytic-like effect, as evidenced by increased percentage of both entries and time spent in open arms. This data is in concordance with other studies which suggest that PDE4 enzyme inhibitors produced anxiolytic activity in EPM test by increasing both number of entries and time spent into the open arms[10,20]. In addition, diazepam used as reference anxiolytic also showed the potential anxiolytic effect in EPM.

L/D test is another widely used animal model for screening anxiolytic or anxiogenic drugs by utilizing the animal's natural preference for dark spaces[21]. The L/D test is designed to exploit the tendency of rodents to explore a novel environment when confronted with the aversive properties of a brightly lit area[22]. In the present study, it was observed that etazolate treatment significantly increased the time spent in and latency time to leave the light compartment. The data in the L/D test is consistent with previous reports which showed that anxiolytic-like effect of a compound in L/D test may be reflected by an increased time spent in and latency time to leave light compartment[23]. Moreover, previous studies have addressed that PDE4 enzyme inhibitor produced anxiolytic-like effects in the L/D test[10]. Furthermore, on the basis of literature the number of transitions between the two compartments is a controversial parameter[21]. Some studies have reported that an anxiolytic drug increase the transitions between the two compartments, while others reported no significant changes in the transition number after treatment with anxiolytic[21]. In the present study, etazolate treatment did not show any effect on the number of transitions between the two compartments.

The anxiolytic-like effect of etazolate was further confirmed by using the HB test. Currently, H-B test is popular as a model of anxiety and offers a simple method for measuring the behavioural response of rodents to an unfamiliar environment[24]. The head dipping behaviour of a rodent in HB is sensitive to change in emotional state of the animal[25]. The results of present study revealed the anxiolytic-like activity of etazolate in HB test, as evidenced by significant increase in the head dipping number and time spent in head dipping. This effect is in agreement with previous studies which suggest that increase in the head dipping number and time spent in head dipping reflects the anxiolytic-like activity of a compound[24,26]. Moreover, in the present study a significant decrease in the head dipping latency of mice was also noted which also indicated the anxiolytic-like activity of etazolate.

The OFT is also widely used for the screening of anxiolytic/anxiogenic drugs. Normal aversion of a rodent to the brightly lit area produces the anxiety and fear, which is characterized by alteration in the behavioural parameters of animal in open field arena. Previous reports suggested that anxiolytic compounds have a tendency to reduce the fearful behaviours of rodents in open field arena[27]. Etazolate treatment increased the ambulation scores and rearing number in OFT indicating the anxiolytic-like effect of etazolate.

Considerable research has been shown that the anxiolytic-like effects of diazepam are mediated through an activation of brain neurotransmitter γ-amino-butyric acid (GABA) at the GABA receptor complex[28]. Although, precise mechanism underlying the anxiolytic effects of etazolate, a PDE4 inhibitor is not fully explored. Numerous studies have reported that the therapeutic response of PDE4 inhibitors in anxiety may be associated with change in cAMP level[29]. A growing body of data has shown that augment cAMP signaling (cAMP/CREB) plays a relevant role in the pathophysiology of anxiety[30,31]. Etazolate induces a significant rise in cAMP level and augments both the intensity and duration of cAMP signaling by inhibiting PDE4 enzyme[13,32]. Moreover, earlier studies have reported a possible relationship between the GABA and cAMP[33]. GABA is also a major neurotransmitter involved in the pathophysiology of anxiety disorder. However, it has been shown that only chronic treatment with PDE4 inhibitor modulates the GABA content[33]. In the present study, the anxiolytic-like effect of acute etazolate (0.25-1 mg/kg, ip) treatment was evaluated. Etazolate at the same dose range produced the antidepressant-like effect by the modulation of cAMP signaling[32]. In this regard, anxiolytic-like effect of etazolate in the present study might be dependent on modulation of cAMP dependent signaling. Further investigations are required to clarify the detailed mechanism(s) of etazolate for anxiolytic-like effect and to find out a correlation between cAMP and GABA in anxiety disorder.

In summary, the results of the present study prove the anxiolytic-like activity of etazolate in four validated animal models of anxiety. Thus, etazolate may offer novel disease modifying and symptomatic

therapeutic potential for the treatment of anxiety disorders with good safety profile with respect to diazepam. However, further studies are needed in this area.

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